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# CHEMICAL AND MOLECULAR BIOLOGICAL ASPECTS OF ALKYLHYDRAZINE-INDUCED CARCINOGENESIS IN HUMAN CELLS IN VITRO

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Metabolic activation of methylhydrazines (MMH, DMH) was studied in cultured human fibroblast cell medium. Binding of DMH and MMH to both cell DNA and protein was observed and quantitated by using radioactive $^{14}C$-methylhydrazines. The cellular binding study will help us to understand the differences in toxicity or carcinogenicity of the various alkyl hydrazines. Synthesis of $^{14}C$-MMH was compled and $^{14}C$-UDMH will be synthesized during the second year. Alkylated DNA was hydrolyzed by using hydrochloric acid

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to the bases and the latter were separated by using HPLC. For MMH and DMH which were non-transformers of human cells, most of the binding was found to be at the phosphodiester oxygens. The binding to the bases was either low (DMH) or absent (MMH). It is hypothesized that the alkylation of phosphodiesters might contribute to the toxicity of these chemicals whereas the alkylation of bases, especially at the O-6 position of guanine, might contribute to their carcinogenicity. Further investigations with $^{14}$C-UDMH will, hopefully, substantiate this hypothesis and studies are in progress towards this goal.

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# CHEMICAL AND MOLECULAR BIOLOGICAL ASPECTS OF ALKYLHYDRAZINE-INDUCED CARCINOGENESIS IN HUMAN CELLS IN VITRO

September 1, 1979 - August 31, 1980

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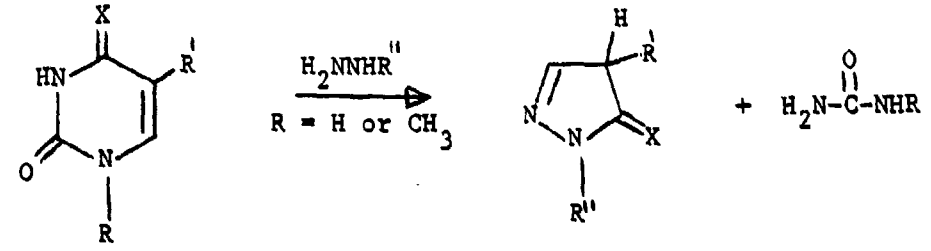
TABLE OF CONTENTS

# Chemical and Molecular Biological Aspects of Alkylhydrazine-induced Carcinogenesis in Human Cells in Vitro

## A. Introduction:

Some carcinogenic alkylating agents such as the N-nitrosoureas are direct-acting and do not require metabolic activation to exert their biological effects. However, many other compounds must be metabolically activated within target tissues to exhibit mutagenicity and/or carcinogenicity. The alkylhydrazines most likely belong to the latter class of compounds, but nucleic acids also are known to react with anhydrous hydrazine and monomethylhydrazine (MMH) but not with phenylhydrazine.[1-5] Pyrimidine bases undergo reaction with hydrazines leading to the formation of pyrazole derivatives and N-alkyl-substituted ureas.




X = O, NH-
R = sugar residue
R' = H, $CH_3$

Such reactions with nucleic acid bases may account for the extensive depolymerization of the polynucleotide chain and hence the mutagenicity of hydrazines, in analogy with proposals suggesting hydroxylamines trigger mutagenic effects via similar reactions.[6] The cause-effect relationship between apyrimidination and carcinogenicity of

hydrazines, however, has not been well studied.

Although hydrazinolysis has been regarded as a pyrimidine-specific reaction, Hayes and Hayes-Baron[4] observed a slow attack of adenine nucleotides by hydrazine hydrate at 90°C. The only degradation product identified was inorganic phosphate.[4] Adenine, guanine and their related nucleosides, as well as guanine nucleotides, were not degraded under similar conditions.[4] Differential loss of adenine residues from ϕx 174 deoxyribonucleic acid on treatment with anhydrous hydrazine was observed by Sedat and Sinsheimer[7] as well as by Ellery and Symons.[8] In view of these observations, it is doubtful that hydrazine, as well as some methylhydrazines, can be considered as truly pyrimidine-specific reagents. The modification of purine as well as pyrimidine bases is thus of significance to this project.

B. Results and Discussion:

1. Metabolism Studies:

Conversion in vitro of various simple monoalkylhydrazines including MMH to their parent hydrocarbons in the presence of rat liver microsomes was reported to require oxygen and a NADPH-regenerating system for maximum activity.[9] Certain N-methylhydrazines including MMH, DMH, and UDMH, were found to be oxidatively demethylated to yield formaldehyde by rat liver microsomal preparations.[10] Again, oxygen and a NADPH-regenerating system were required for maximum activity.[10] Further studies revealed that the alkylhydrazine oxidase activity was not dependent upon cytochrome P-450 and was not inducible by prior treatment of rats with either phenobarbital or 3-methylcholanthrene.[11] N-methylhydrazine demethylase activity, however, was

inducible in rats and the induced activity appeared to be dependent upon the P-450 content of the microsomes. Non-induced rats possessed demethylase activity which did not appear to be P-450 dependent.[11] It was concluded that there are two enzyme systems present in rat liver microsomes capable of demethylating methylhydrazines.[11]

To our knowledge, microsome-mediated modification of macromolecules by methylhydrazines in vitro has not been investigated. Although not unequivocal, it is widely believed that alkylation of biomacromolecules such as DNA or proteins might play an important role in the process of chemical carcinogenesis.[12,13] We have developed an in vitro rat liver microsome-mediated DNA methylation system in which DMH functioned as the methylating agent. Control experiments showed an absolute requirement for NADPH as well as for microsomal enzymes. The liver microsomes used in this study were obtained from male Sprague-Dawley rats pretreated with phenobarbital. The incubation medium for the in vitro methylation of the exogenously added calf thymus DNA contained sodium phosphate buffer (pH=7.4), EDTA, NADPH, $MgCl_2$ and rat liver microsomal protein (S-9 mix) and $^{14}C$-DMH (sp. activity 6 mCi/mmol). After incubation at 37°C for 60 min, DNA and protein were separated and analyzed for radioactivity. The experiment was run in triplicate to obtain the average values. The radioactivity found was 2900 dpm per mg of DNA and 11,800 dpm per mg of protein. To quantify the methylated bases, DNA was hydrolyzed with 0.1 M HCl at 37°C for 24 hrs. 3-Methyladenine, 7-methylguanine and O-6-methylguanine were added in nanogram quantities (a stock solution containing the mixture of these bases in 0.01N hydrochloric

acid was stable for several weeks when kept in the refrigerator) to the hydrolyzed DNA. The pH was adjusted to 3, and the mixture was loaded onto a column of Sephadex G-10 (80 x 1.6 cm) and eluted with ammonium formate buffer (0.05 M, pH=6.8) at 1 ml $min^{-1}$. The optical density (254 nm) was monitored via a flow cell and 4 ml fractions were collected. Fractions 14-22 contained the apurinic acid; fractions 34-37 contained the 3-methyladenine (3-MeA); 57-70 contained 7-methylguanine (7-MeG) followed by guanine (78-87), adenine (94-104) and finally 6-O-methylguanine (6-OMeG) (120-140). The fractions corresponding to each peak were combined and evaporated to a final volume of 1.5-2 ml. Biofluor (10-18 ml) was added and the mixture was shaken and placed in the refrigerator. The resulting homogenous solution was counted for radioactivity. The counting efficiency was 75% and the cpm values were converted to dpm's. Table 1 illustrates the extent of alkylation of the exogenously added calf thymus DNA and the endogenous microsomal protein expressed as disintegrations per minute (dpm).

Table 1. Methylation of protein and exogenously added DNA by 1,2-DMH ($^{14}C$) in the presence of rat liver S-9 mix and NADPH.

| Expt. | dpm/mg protein | | dpm/mg DNA | |
|---|---|---|---|---|
| (1) | 11,560 | | 2875 | |
| (2) | 12,100 | average | 2960 | average |
| (3) | 11,740 | 11,800 | 2865 | 2900 |

Table 2 illustrates the binding of $^{14}C$-DMH to the bases of DNA as

well as to the apurinic acid. It is clear that most of the radioactivity (78.6% of the total activity per mg DNA) was associated with the apurinic acid whereas only 14.1% of the activity was observed as methylated bases.

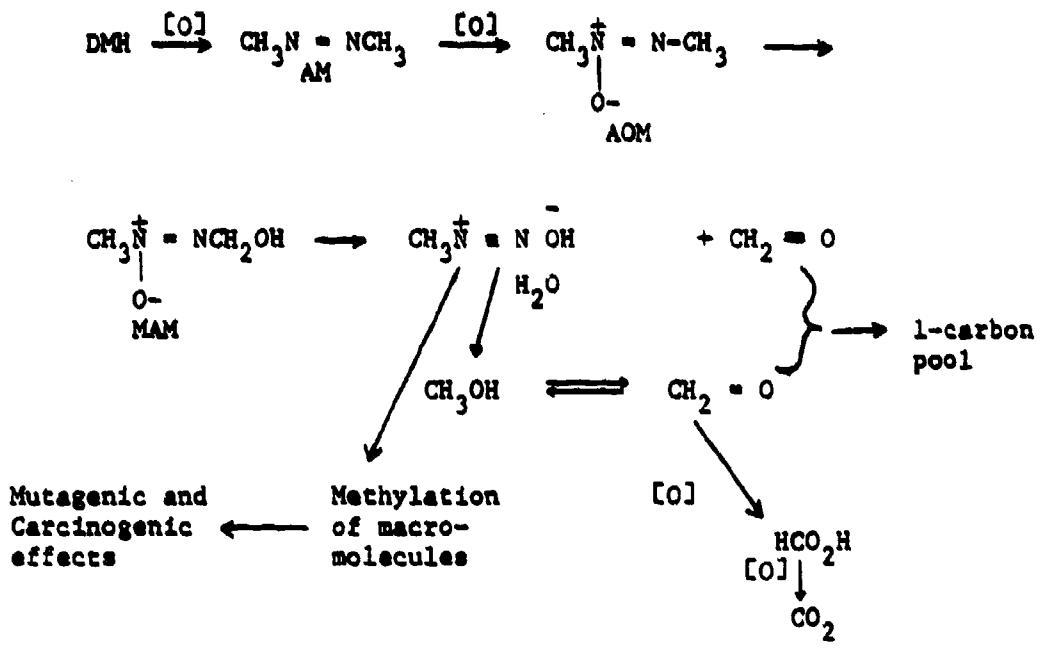
Table 2. Binding of $^{14}$C-DMH to bases and apurinic acid of DNA (1 mg).

| Fractions | Activity (dpm) | % of total radioactivity found in DNA |
|---|---|---|
| 1) Apurinic acid | 2280 | 78.6 |
| 2) N-7-Methylguanine | 145 | 5.0 |
| 3) 3-Methyladenine and 1-Methyladenine | 200 | 6.9 |
| 4) 6-O-Methylguanine | 65 | 2.2 |

The ratio of 6-OMeG to 7-MeG was 0.45.

Binding of Alkylhydrazines to Biomacromolecules in Human Fibroblast Tissue Culture.

In addition to the rat liver microsome-mediated metabolism studies using DMH[9-11] animal studies dealing with metabolic activation of DMH have been reported by many groups.[14-17] DMH has been shown to follow a metabolic pathway similar to that of dimethyl nitrosoamine.[15] The following scheme illustrates the oxidative metabolism of DMH.




The electrophile, methyl diazonium cation, may react with cellular nucleophiles (DNA, RNA, Proteins) to yield methylated macromolecules or react with water to form methanol which in turn may be oxidized to formaldehyde by alcohol dehydrogenase.[18] In the case of radioactive DMH, the labeled formaldehyde is expected to enter the one-carbon pool and incorporate into tissue constituents via normal biosynthetic pathways. Alternatively, formaldehyde could be oxidized to formic acid and then to carbon dioxide. These processes have found parallels in the metabolic activation of dialkylnitrosamines.[19]

Metabolic activation of DMH in cultured human colon epithelia and binding of the metabolites to both cell DNA and protein was reported by Autrup et al.[20] $^{14}$C-DMH was shown to methylate DNA at both the O-6 and N-7 positions of guanine and at the N-3 position of adenine. Also, de novo synthesis of adenine and guanine was observed.

Formation of $^{14}CO_2$ was observed but not quantitated. The ratio between methylation of C-6 and N-7 positions was 0.5. Also, considerable activity was associated with the apurinic acid, but the extent of methylation was not measured. Studies by Milo et al.[21] have revealed that in human fibroblast cell cultures, Hz and UDMH induce neoplastic transformation, whereas MMH and DMH only elicited cellular toxicity. Also, Hz and UDMH serve as cocarcinogens in the FeSV-induced transformation system.

To understand differences in toxicity or carcinogenicity relative to binding profiles cellular binding studies were undertaken in these laboratories using radioactive alkylhydrazines. We anticipate that these studies using the human fibroblast cell system would compliment studies with human colon epithelial cells carried out by Autrup et al.[20]

The binding levels of $^{14}$C-DMH to both DNA and protein in human fibroblast cells are shown in Table 3. Table 4 gives details of the methylation pattern of the bases in DNA.

Table 3. Binding Data for $^{14}$C-DMH to Cell DNA and Protein in Human Fibroblast Cell Culture.

| | DNA | | PROTEIN | |
|---|---|---|---|---|
| Expt. | Radioactivity per mg DNA dpm | Picomoles of DMH bound per mg DNA | Radioactivity per mg protein dpm | Picomoles of DMH bound per mg protein |
| 1 | 35,750 | 2684 | 8350 | 627 |
| 2 | 34,960 | 2625 | 8438 | 634 |
| 3 | 34,470 | 2588 | 8410 | 631 |
| Avg. | 35,060 ±690[a] | 2632 ±52 | 8399 ±49 | 631 ±4 |

[a]Standard deviation

Table 4. Binding of $^{14}$C-DMH to the Purine Bases of DNA and to Apurinic Acid.[a]

| Fractions | Radioactivity dpm | Picomoles DMH bound to each fraction |
|---|---|---|
| Apurinic Acid | 30,300 ±510[b] | 2275 ±35[b] |
| N-7-Methylguanine | 940 ±12 | 71 ±2 |
| O-6-Methylguanine | 477 ±7 | 36 ±1 |
| Methyladenines (3- and 1-) | 1,000 ±15 | 75 ±2 |
| Adenine | 898 ±13 | 67 ±2 |
| Guanine | 884 ±12 | 66 ±2 |

[a]Each number is an average of 3 values. [b]Standard deviation.

In addition to measurement of radioactivity associated with DNA and protein, we were able to trap the $CO_2$ liberated from the incubation mixture using hyamine hydroxide solution. For each experiment, using 20 μmol of $^{14}$C-DMH (specific activity 6 mCi per mmol), the liberated $CO_2$ had an activity of 8,463,280 dpm which amounted to 635 nmol of $CO_2$. Thus, the radioactivity found in the unmethylated bases (adenine and guanine) likely reflect incorporation of $^{14}$C from the 1-carbon pool.

There are some significant differences as well as similarities between our binding data (using human fibroblasts cells) and data reported using human colon epithelia.[20] The absolute amount of DMH bound to DNA was approximately 100-fold higher in our studies when compared to those using epithelial cells.[20] Binding to protein was

only lower by a factor of 3-10, when compared to the published data using colon epithelial cells.[20] Regarding methylation of bases, methylation at the N-7 and O-6 positions of guanine was nearly identical in both studies. The ratio of O-6 to N-7 methylation was 0.507 in our study and 0.500 in the reported study with epithelial cells.[20,22] The amount of methylation at the 3- and 1-positions of adenine [3-Methyl and 1-Methyladenines could not be separated using Sephadex G-10 column chromatography, but could be separated using a Partisil PXS 10/25 SCX HPLC column (see Materials and Methods Section)] was markedly increased when compared to Autrup's study[20] (1000 dpm vs 178 dpm). Similarly, the extent of incorporation of $^{14}$C-DMH metabolites into purine rings of nucleic acids by de novo synthesis was higher in our system than in the colon epithelia cell system (adenine, 898 vs 241 dpm; guanine, 884 vs 222 dpm). Another major difference in these two studies lies in the percent activity found in the bases relative to DNA. In our studies, the total activity of the bases accounted for 12.0% of the DNA radioactivity prior hydrolysis, whereas in the case of epithelial cells the bases accounted for 32.8% of the DNA radioactivity.[20] Most of the activity of DNA (86.4%) resided in

Table 5. Binding Data for $^{14}$C-MMH to Cell DNA and Protein in Human Fibroblast Cell Culture.

| | DNA | | PROTEIN | |
|---|---|---|---|---|
| Expt. | Radioactivity per mg DNA dpm | Picomoles of MMH bound per mg DNA | Radioactivity per mg protein dpm | Picomoles of DMH bound per mg protein |
| 1 | 15,860 | 121 | 2100 | 16 |
| 2 | 14,650 | 112 | 2650 | 20 |
| Avg. | 15,255 ±615 | 116.5 ±4.5 | 2375 ±275 | 18 ±2 |

Table 6. Binding of $^{14}$C-MMH to the Purine Bases of DNA and to Apurinic Acid.[a]

| Fractions | Radioactivity dpm | Picomoles of MMH bound to each fraction |
|---|---|---|
| Apurinic Acid | 8,420 ±120[b] | 64 ±2 |
| Guanine | 3,250 ±52 | 25 ±1 |
| Adenine | 3,100 ±50 | 24 ±1 |

[a]Each determination is an average of two values. [b]Standard deviation.

the apurinic acid fraction which consisted of phosphotriesters and pyrimidine oligonucleotides. Singer[23] observed high alkylation (70% of total DNA radioactivity) of the phosphodiesters resulting from alkylation in vitro of Hela cell DNA by ethylnitrosourea, a potent carcinogen that does not need metabolic activation to exert its activity.

The significance of the formation of large amounts of phosphotriesters in relation to mutagenesis or carcinogenesis is not clear. It may be that alkylation of phosphodiesters of DNA contributes to the cellular toxicity of the parent chemical tested. This hypothesis is in accord with the findings of Milo et al.[21] showing that DMH and MMH elicit only cellular toxicity, but not transformation in the fibroblast cell system.

Tables 5 and 6 deal with the binding data for $^{14}$C-MMH in human fibroblast cells. In general, the binding levels for MMH were lower than those for DMH. The amount of $CO_2$ liberated per µmol of MMH used was only 247 pmol and this was 129 times less than the amount of $CO_2$

liberated per μmol of DMH. Nearly all the activity in DNA resided in the apurinic acid and in the purine bases formed via de nova synthesis. There was no detectable amount of methylated bases present in the DNA hydrolysate.

Table 7 provides data regarding the toxicity, transformation potentials and the tumor-incidence by various hydrazines and some of their metabolites. It must be pointed out that DMH did not transform cells in the human fibroblast system but its metabolite, methylazoxymethanol acetate (MAMA), proved to be a potent transformer as well as a carcinogen. The obvious conclusion is that the human cell culture used in our study did not provide enzymes capable of metabolizing DMH to the critical intermediates necessary for cell transformation.

Table 7. Toxicity, Transformation Potential, and Tumor Incidence Data for Hydrazine, Methylhydrazines their Presumed Metabolites, and Phenylhydrazine.

| Compound | E.D. 50 | Boluses Frequency[a] | Tumor Incidence[b] |
|---|---|---|---|
| Hydrazine | 35 μg/ml | 9.2 | 4/8 |
| UDMH | Negative at 100 μg/ml | 5.1 | 4/8 |
| DMH | Negative at 100 μg/ml | Negative | ——— |
| MMH | 62 μg/ml | Negative | ——— |
| AOM | Negative at 100 μg/ml | Not tested | ——— |
| MAMA | 3.6 μg/ml | 900 | 2/16[c] |
| Tetrazine | 50 μg/ml | Positive. No data yet. | ——— |
| Phenylhydrazine | 16 μg/ml | Negative | ——— |

[a]Number of boluses per 100,000 cells seeded into 0.33% agar.

[b]$5 \times 10^6$ cells were injected S.C. into nude mice. Tumors developed within 2-4 weeks. [c]Some animals died during the expt. Also, the ages of the animals were not the same as those of the mice used for UDMH and Hz. Hence, the tumor incidence data for MAMA is not comparable to those of UDMH and Hz.

C. Conclusion:

In our attempts to correlate toxicity and/or carcinogenicity of methylhydrazines with alkylation of the phosphodiesters and/or the purine bases, we have carried out the analysis of the alkylated DNA-hydrolysate. For the non-transforming (in human fibroblast cells) methylhydrazines (MMH and DMH) methylation of purine bases were absent or low, respectively. However, there was considerable binding to the phosphodiester oxygens. The latter event may be related to cell toxicity, whereas the DNA-alkylation of base may be related to the transformation event. Further investigations planned using UDMH should enable us to provide information concerning this proposal since UDMH does transform the human fibroblast cell line.

D. Materials and Methods:

1. Chemicals:

$^{14}$C-DMH (specific activity, 6mCi per mmol) was purchased from New England Nuclear (Boston, Ma.) and the unlabeled methylhydrazines, MMH, UDMH, and DMH were purchased from Aldrich (Milwaukee, Wis.) and distilled prior to use. The purities of the samples were routinely checked by the TLC as described by Fiala and Weisburger.[24] Calf thymus DNA, pronase, and ribonuclease A were obtained from Sigma (St. Louis, Mo.). Ribonuclease was preheated at 80°C for 30 min in 0.1 x SSC buffer (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) and refrigerated prior to use. 7-Methylguanine, 3- and 1-methyladenines, guanine and adenine were purchased from either Sigma or Vega (Tucson, Az.). O-6-Methylguanine was synthesized according to a procedure described by Balsinger and Montgomery.[25] The presumed metabolites

azoxymethane (AOM) and methylazoxymethanol (acetate form) (MAMA) were purchased from Sigma Chemical Co. Tetramethyl-2-tetrazene was synthesized by HgO oxidation of UDMH.[26] The experimental methodology for synthesis of $^{14}$C-MMH is described later in this report.

Rat liver S-9 fractions were obtained from phenobarbital-pretreated (ip dose of 100 mg per Kg body weight per day, 4 days) male Sprague Dawley rats. The animals were killed by decapitation, the livers were removed and homogenized with 20 ml ice-cold potassium chloride. After centrifuging the homogenate at 9,000 x g for 60 min, the supernatant S-9 mix was removed and stored at -70°C.

2. Instruments:

Laboratory Data Control (LDC) chromatography accessory module containing LDC gradient master, constametric pumps, spectromonitor III and equipped with Whatman Partisil PXS 10/25 SCX Column (length 25 cm; diameter 4.6 mm) and a Whatman Precolumn were employed for high pressure liquid chromatography (hplc) purposes. The column was eluted with 0.2 M sodium phosphate buffer (pH 3.0) at a flow rate of 0.64 ml $min^{-1}$. The detector wavelength was set at 280 nm. The retention times of the bases and apurinic acid are as follows: Apurinic Acid, 3.7 min; Guanine, 7.8 min; Adenine, 14.4 min; 7-Methylguanine, 18 min; 1-Methyladenine, 29 min; O-6-Methylguanine, 35 min; and 3-Methyladenine, 50 min. The fractions were collected every two minutes and mixed with scintillation cocktail (Biofluor, New England Nuclear) (10-15 ml) and cooled to obtain a clear solution whose radioactivity was measured. The counting efficiency was 75% as determined by external standard calibration methodology.

Rat liver microsome-mediated in vitro binding of $^{14}$C-DMH to exogenous DNA and endogenous microsomal proteins.

The reaction system (incubation mixture) contained calf thymus DNA (2 mg), EDTA (150 µmol), magnesium chloride (2 µmol), NADPH (3 mg, Sigma), sodium phosphate buffer (pH 7.4, 0.1 M, 1.5 ml), microsomal protein (7.5 mg), and $^{14}$C-DMH (0.51 µmol, spec. act. 6 mCi/mmol). Incubation was carried out at 37°C for 60 min. Three ml of Kirby's phenol reagent (75 ml m-cresol; 0.5g 8-hydroxyquinoline, and 600g of phenol in 55 ml water) was added, vortexed for 5 min, and centrifuged at 10,000 x g for 30 min. The upper layer was removed by pipet and the phenol layer was extracted with 4 ml of sodium phosphate buffer (vortex and centrifuge). The top layer was removed and to the pooled aqueous layers was added absolute ethanol (20 ml). After standing in the refrigerator overnight, the precipitated DNA was centrifuged (10,000 x g; 20 min) and the pellet was washed with ethanol (2 x 10 ml) and ether (2 x 10 ml). The pellet was dried under $N_2$, redissolved in 3 ml of 0.1 x SSC buffer (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), and treated with preheated ribonuclease (150 µg) for 30 min at 37°C. After subsequent treatment with pronase (200 µg) for 60 min at 37°C the DNA was isolated using one extraction with Kirby's phenol (3 ml). The extract was treated with ethanol (20 ml) and DNA. The pellet (ppt) was dissolved in double-distilled water (1 ml). Aliquots of this solution were taken for DNA analysis and radioactivity measurements.

The initial phenol layer was mixed with 10 ml of ethanol and the precipitated proteins were chilled at 0°C for 48 hr, centrifuged at

10,000 x g for 20 min, washed with ethanol (10 ml) and ether (2 x 10 ml), dried by blowing $N_2$ and dissolved in 10 ml of 0.1N NaOH solution. Aliquots of the solution were used for the Bio-Rad assay of protein and for measurement of radioactivity.

General Methodology for the Treatment of Human Fibroblast Cells with Radioactive Methylhydrazines. Biochemical Analysis of the Treated Cells.

Human neonatal foreskin fibroblasts were grown in minimal essential medium (Eagle's) - 25.0 mm HEPES, pH 7.2, containing 2.0 mm glutamine, 1x non-essential amino acids, 1x sodium pyruvate, 0.5 µg per ml gentamicin, 25 mm sodium bicarbonate, and 10% fetal bovine serum (FBS). The cells were routinely passaged 1 to 10 in this medium, seeded into 150 mm tissue culture dishes and placed in a 4% $CO_2$, high humidity incubator. When at a 70-80% confluent density, the cells (PDL < 10) were treated with the radioactive methylhydrazine (DMH or MMH) in 10 ml of the above medium but with 2% FBS. After 24 hrs, the treated medium was removed, the cells washed with incomplete medium (minus FBS) and harvested by scraping with a rubber policeman. After pelleting by centrifugation, the cells were frozen in liquid nitrogen and stored at -20°C until the biochemical analysis was carried out.

To the total cell lysate in a 50 ml Sorvall tube was added 4 ml of the 0.1 x SSC buffer, 400 µl of 10% SDS solution and 3 ml of Kirby's phenol. The mixture was vigorously vortexed for 5 min and centrifuged at 10,000 x g for 30 min. The aqueous layer was carefully removed by pipet and the phenol layer was extracted with 4 ml of 0.1 x SSC

buffer (vortex and centrifuge). The aqueous layer was used for the precipitation and purification of DNA and the phenol layer was used for the precipitation and purification of proteins as described in the microsome-mediated alkylation experiment. Protein analysis was carried out by Bio-Rad assay and the DNA assay was carried out by the UV absorptions at 260 and 280 nm according to the following equation:

$$\mu gDNA/ml = \frac{A_{260}-A_{280}}{0.01}$$

The DNA solution in water was adjusted to pH 1 by the addition of 1N HCl and heated at 70°C for 30 min. Methylated bases were added and the mixture was analyzed either by Sephadex G-10 column or preferably by HPLC as described in an earlier section.

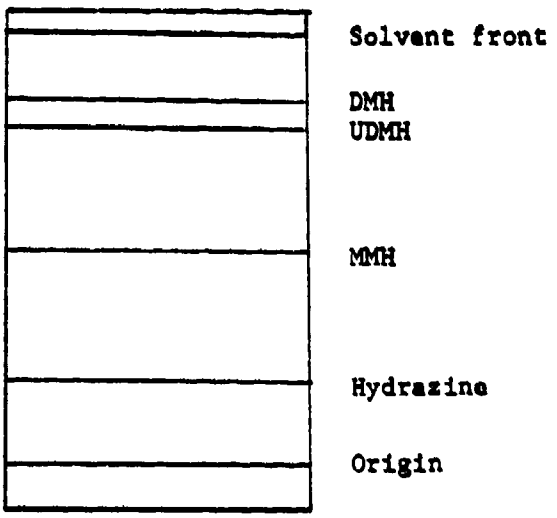
E. Synthetic Methodology

Synthetic efforts during the last several months have focused primarily on the synthesis of radiolabelled $^{14}C$-dimethylhydrazines. The labelling synthesis, (while seemingly elementary) has inherent limitations owing to the very small quantity (20 µl) of labelled starting material involved. Moreover, both isomers (i.e., 1,1-dimethyl- and 1, 2-dimethylhydrazine) have similar physical properties and are not easily separated into pure entities. Absolutely pure materials are required for our biological studies.

Preliminary experiments were also performed for the synthesis of certain metabolites. Preliminary cold runs have been worked out and radiolabelled synthesis for azoxymethane and azomethane are planned for the future.

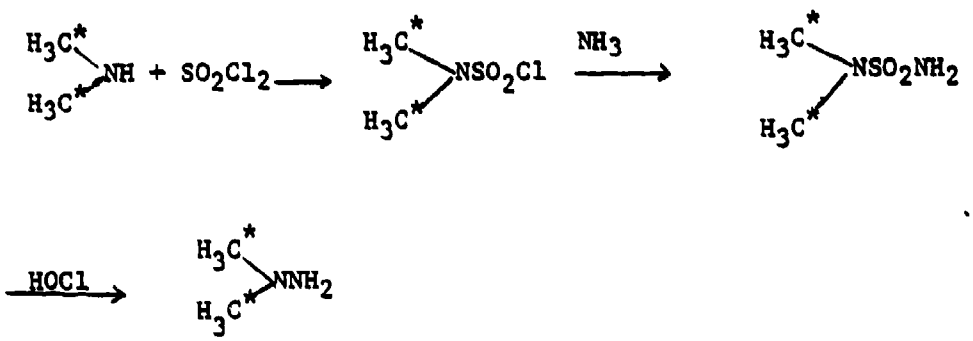
1. Monomethylhydrazine: (Tris)-trimethylsilyl hydrazine was utilized as starting material, and was prepared according to procedures published by Wannagat and Co-worker.[27] The tris (trimethylsilyl)hydrazine anion was generated in situ using n-BuLi in $Et_2O$ and allowed to undergo reaction with $^*CH_3I$ yielding monomethylhydrazinehydrochloride following acidic hydrolysis. Experimental details were provided in our previous report.

To be certain that MMH was not contaminated by any other radiochemical, analytic TLC of the radiolabeled sample was carried out along with chromatography of authentic cold MMH.HCl. After chromatographic development, the plate was sprayed with Folin-Ciocalteau reagent which afforded a blue color at the site of the cold MMH spot. The corresponding radiolabelled area was scraped off and counted. The areas corresponding to dimethylhydrazine were also scraped off and checked for possible contamination.

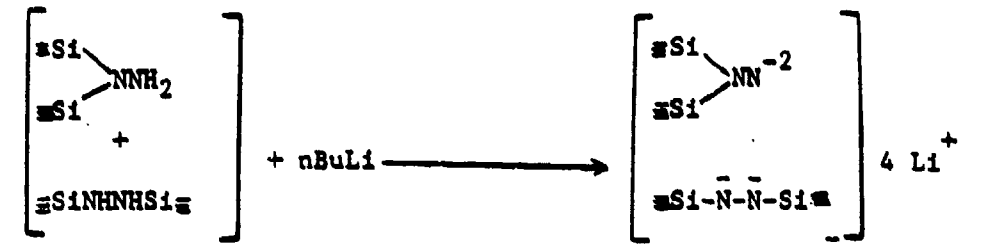


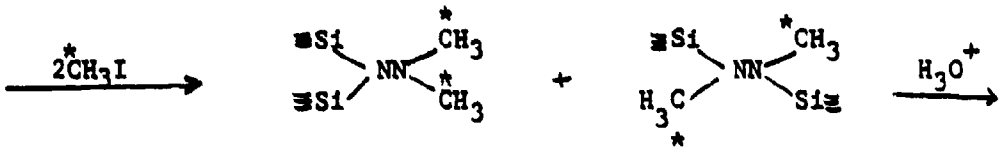

2. Dimethylhydrazines: Since $^{*}CH_3I$ is the only available radiolabelled starting material of high specific activity, the synthetic scheme has to be adjusted according to this availability.

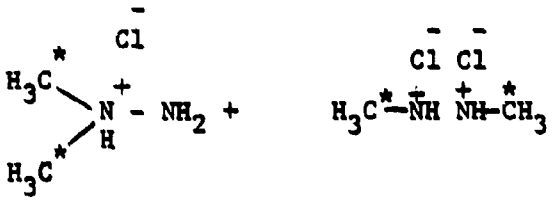
As mentioned in our previous report, dimethylamine might represent another possible starting radiochemical for the synthesis of UDMH according to the following scheme. However, commercially available dimethylamine has only a rather low specific activity (5-10 mci/mm).



Another route may utilize N-nitrosomethylamine (not commercially available) in reaction with $^{14}CH_3I$. This route likely has no advantages over the silylhydrazine route described subsequently. This methodology would only yield UDMH after hydrogenation and a second scheme would have to be devised for the preparation of DMH of high specific activity. Furthermore, DMH of high specific activity which in turn will serve as radiolabelled starting material for Azo- or Azoxymethane and MAMA-metabolites. The following synthetic strategy are being used to prepare radiolabelled DMH and UDMH.




sp. activity ~ 100 mCi/mmole

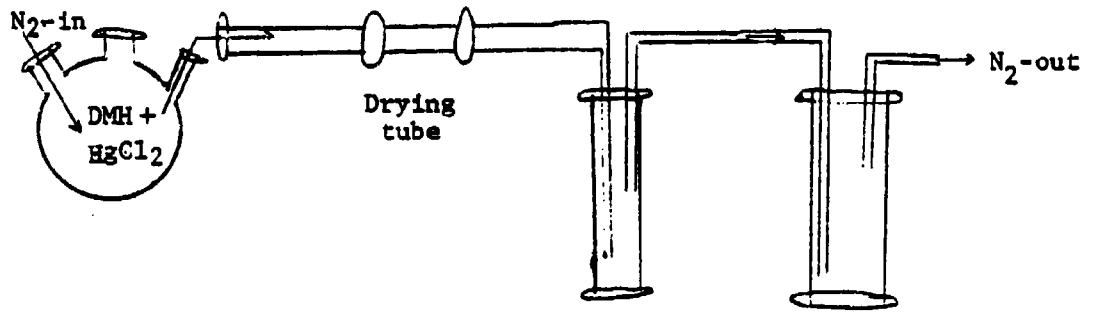
Advantages associated with this scheme include: (1) Bissilyl-hydrazines are stable nonvolatile starting materials, and (2) A 50/50 mixture of radiolabeled DMH and UDMH are obtained; both compounds are useful to this research. Modification of the original solvent system from (65:15:30) i-Propanol:HCl:water to (75:17.5:7.5) allows for separation of DMH and UDMH on an analytical TLC plate. Preparatively, this solvent system fails to separate the mixture. Further work is in progress to resolve this problem. HPLC may be employed, but also has disadvantages. Fiala[28] reported that DMH binds to HPLC columns strongly and can only be eluted with acidic solvent (0.05M acetic acid). Moreover, after separation, one must remove large amounts of acetic acid from small quantities (3-4 mg) of hydrazine salts.

Separation based on chemical derivatization seems to us to be the better choice. UDMH forms hydrazones with a variety of aldehydes in >95% yield. These 1,1-dimethylhydrazones can be exchanged with hydrazine to form hydrazine-hydrazone and free UDMH. When p-nitrobenzaldehyde underwent reaction with UDMH:HCl in ethanol, UDMH hydrazone was obtained after ~ 15 minutes at RT. The yield of solid hydrazone was ~98% and could easily be separated from liquid. The exhange reaction with hydrazine proceeded smoothly at RT and solid hydrazine-hydrazone was formed from which 1,1-dimethylhydrazine and unreacted hydrazine were removed by distilation. Since the exchange reaction proceeds in only 50% yield, we are currently investigating utilization of benzaldehyde which

is also reported to form hydrazones with UDMH in greater than 90% yield. No problem is anticipated when separating UDMH from unreacted hydrazine by preparative TLC since these two substances are easily resolved.

3. Synthesis of Metabolites: The proposed mechanism of metabolism for DMH involves formation of AM, AOM, MAM and possible hydrolysis to methyldiazonium alkylating species. Thus, we are presently oxidizing[29] DMH (free base) using yellow $HgCl_2$. Azomethane gas formed is trapped in m-chloroperbenzoic acid/ether solution at -78°C. This reagent oxidizes the azomethane to azoxymethane (liquid, bp. 98°C) which in turn was isolated or may serve as starting material for the acetate derivative MAMA (a potent transforming agent).

Use of radiolabeled DMH is anticipated to yield desired radiolabeled metabolites required for biological studies.




m-chloroperbenzoic Acid/ether solution at -40°C

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